Marine Biology

Molecular identification of three co-occurring species of Acartia in the Thau lagoon, France.

--Manuscript Draft--

Manuscript Number:	
Full Title:	Molecular identification of three co-occurring species of Acartia in the Thau lagoon, France.
Article Type:	Method
Keywords:	Acartia; Thau lagoon; resting eggs; PCR-RFLP
Corresponding Author:	Penelope Kate Lindeque, PhD Plymouth Marine Laboratory Plymouth, UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Plymouth Marine Laboratory
Corresponding Author's Secondary Institution:	
First Author:	Penelope Kate Lindeque, PhD
First Author Secondary Information:	
Order of Authors:	Penelope Kate Lindeque, PhD
	Delphine Bonnet, PhD
	Séverine Boyer
Order of Authors Secondary Information:	
Abstract:	Acartia species, often known to co-occur, can exhibit complex life cycles, such as the production of resting eggs. Studying and understanding their population dynamics is hindered by the inability to identify eggs and early developmental stages using morphological techniques. A simple molecular technique to distinguish between the three species of the Acartia genus (A. clausi, A. discaudata and Paracartia grani) that co-occur in the Thau lagoon (40°03 N; 03°41 E) in Southern France has been developed. Direct amplification of a partial region of the mitochondrial cytochrome oxidase I (mtCOI) gene by polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism (RFLP) results in a unique restriction profile for each species. The technique is capable of determining the identity of individual copepods and eggs, including resting eggs retrieved from sediment samples, illustrating its application in facilitating population dynamic studies of this ubiquitous and important member of the zooplankton community.
Suggested Reviewers:	Ann Bucklin University of Connecticut ann.bucklin@uconn.edu Leading researcher in this area. If busy may be able to suggest alternative reviewer eg Leocadio Blanco-Bercial, Jasmin Renz or Sabrine Grabbert. All have published in this area
	Lidia Yebra, PhD Instituto Espanol de Oceanografia lidia.yebra@ma.ieo.es Zooplankton ecologist with molecular biology experience
	Jasmin Renz, PhD Forschungsinstitut Senckenberg jrenz@senckenburg.de Previously published in this area

Molecular identification of three co-occurring species of *Acartia* in the Thau Lagoon, France.

P. K. Lindeque[1], S. Boyer[2] and D. Bonnet[2].

1. Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth, PL1 3DH, UK.
pkw@pml.ac.uk, Tel: +44 (0)1752 633100, Fax: +44 (0)1752 633101.
2. Laboratoire EcoSym (Ecologie des Systèmes Marins Côtiers), UMR5119, Université Montpellier 2, CC093, Place Eugène Bataillon 34095 Montpellier Cedex 05, France.

Key Words: Acartia, Thau lagoon, resting eggs, PCR-RFLP

Abstract

Acartia species, often known to co-occur, can exhibit complex life cycles, such as the production of resting eggs. Studying and understanding their population dynamics is hindered by the inability to identify eggs and early developmental stages using morphological techniques. A simple molecular technique to distinguish between the three species of the *Acartia* genus (*A. clausi*, *A. discaudata and Paracartia grani*) that co-occur in the Thau lagoon (40°03 N; 03°41 E) in Southern France has been developed. Direct amplification of a partial region of the mitochondrial cytochrome oxidase I (mtCOI) gene by polymerase chain reaction (PCR) and subsequent restriction fragment length polymorphism (RFLP) results in a unique restriction profile for each species. The technique is capable of determining the identity of individual copepods and eggs, including resting eggs retrieved from sediment samples, illustrating its application in facilitating population dynamic studies of this ubiquitous and important member of the zooplankton community.

Introduction

The copepods from the Acartiidae family generally occur in estuaries, harbours and other semi enclosed marine habitats (Alcaraz 1983), in which food is rarely limited (Calbet *et al*. 1999). *Acartia* species are commonly known to co-occur in the same environment and in Mediterranean bays, lagoons and harbours, the number of species reported to co-exist varies from two in the ports of Naples (Yamazi 1964) and Marseille (Arfi *et al*. 1981) to six in the small port of Jounieh (Lakkis 1994). In the Thau lagoon, the largest of a string of lagoons that stretch along the Languedoc-Roussillon French coast from the Rhône River to the foothills of the Pyrenees, the genus *Acartia* represents the most abundant taxon in the plankton community (Fatemi 1938; Lam Haoi 1985). To our knowledge, 5 congeneric species have been identified until now in the Thau lagoon: *A. clausi*, *A. bifilosa*, *A. discaudata*, *A. margalefi* and *A. latisetosa* (Mathias and Euzet 1962; Lam Haoi 1985). However, unpublished observations (Euzet pers comm; Boyer *et al*. in press) have highlighted that since 1998, *Paracartia grani* has appeared in this lagoon. A two year monitoring program in the Thau lagoon (40°03' N; 03°41' E), has shown that *A. clausi* predominates in winter, while *A. discaudata* peaks in spring and *P. grani* in summer (Boyer *et al.* submitted).

Acartia species are known to have complex life cycles with *A. clausi* and *P. grani* producing resting eggs (i.e. Kasahara *et al*. 1974; Marcus 1990; Guerrero and Rodriguez 1998). To study the population dynamics of these species, abundance, stage development and egg production of *Acartia* spp. have been recorded every two weeks during a 2 year survey in the Thau lagoon (Boyer *et al*. submitted). Sediment cores were also collected at two dates (May 2010 and April 2011) to investigate the potential pool of recruits coming from the sediment. In 2010, copepod eggs extracted from the sediment were incubated and a very high hatching success was observed. To establish the species identity of the eggs, they were maintained individually in an attempt to allow them to reach a developmental stage suitable

for morphologic identification. Unfortunately, this was never successful as many nauplii and juveniles died before reaching copepodite stages.

In situations where morphological characters do not provide sufficient variation to identify to species level, as is the case with the resting eggs and early developmental stages of *Acartia* spp., then taxonomic discrimination can be achieved using genetic characters. Such techniques have been developed for copepods of the genus *Pseudocalanus* (Bucklin *et al*. 1998; Grabbert *et al*. 2010), *Calanus* (Lindeque *et al*. 1999; Bucklin *et al*. 1999; Hill *et al*. 2001) and *Clausocalanus* (Blanco-Bercial and Alvarez-Marques 2007). However, no such technique has been developed for *Acartia* species. Therefore, during this study we developed a molecular tool to identify individuals, from eggs to adults of the three congeneric species, *A. clausi, A. discaudata, and P. grani*, to facilitate population dynamics studies. These three species were chosen as they are by far the dominant co-occurring species (Lam Haoi 1985; Boyer *et al*. submitted) and because two of them (*A. clausi* and *P. grani*) are known to produce resting eggs, an important life strategy for surviving adverse periods (Kasahara *et al*. 1974; Marcus 1990; Guerrero and Rodríguez 1998). The technique therefore, has to be sufficiently robust to work on individual eggs, including resting eggs taken from sediment cores.

The gene of choice for our study was the common metazoan DNA barcoding gene, mitochondrial cytochrome oxidase subunit I (mtCOI). This gene has previously been used for the identification and assessment of species diversity and distribution of marine zooplankton (Bucklin *et al*. 1999; Hill *et al*. 2001; Webb *et al*. 2006; Blanco-Bercial and Alvarez-Marques 2007; Grabbert *et al*. 2010; Bucklin *et al*. 2010). However, at the beginning of this study, out of the three species *A. clausi, A. discaudata* and *P. grani*, only sequence data for the mtCOI gene for *A. clausi* existed on the EMBL DNA sequence database. Therefore, a sequencing effort of adults of all three species collected from the Thau lagoon was necessary. The use of

a mitochondrial gene was preferential as such a gene has a high copy number and is relatively accessible to PCR primers allowing PCR amplification to be achieved direct from an individual egg or copepod without prior purification of the DNA. The mitochondrial 16S rRNA gene, which has previously been used for copepod species identification (Bucklin *et al*. 1998; Lindeque *et al*. 1999) showed insufficient inter-specific variation between the *Acartia* species (Lindeque unpubl data).

A simple and unambiguous method to distinguish between the co-occurring *Acartia* spp. in the Thau lagoon, including early developmental stages and resting eggs, will allow a better description and understanding of the species population dynamics.

Material and Methods

Sample collection and preservation

Zooplankton samples were collected at a fixed station (40°03 N; 03°41 E) close to Sète channel in the Thau lagoon (south of France). Horizontal hauls were performed in the inner surface water (maximum 1 m depth) with a WP2 plankton net (200-µm mesh size). The cod-end contents were placed in insulated containers and brought back to the laboratory within 1 h after collection. In the laboratory, healthy females of *A. clausi*, *A. discaudata* and *P. grani* were picked out. Physiological state of the female (ovigerous versus non ovigerous) was determined with a binocular microscope using morphological characteristics (Rose 1933). For each species, ovigerous females were incubated for 24 h in a 1 L beaker filled with 0.45 µm filtered sea water, after which the females and the eggs produced were fixed in 95% alcohol.

Two sediment cores were sampled on the 27th of May 2010 and the 27th April 2011 at the same monitoring station by a diver. The cores were sliced and each slice preserved at 4°C

in darkness before analyses. Resting eggs were extracted from the sediment samples according to the method of Onbé (1978). In 2010, copepod eggs extracted from the sediment were used for hatching experiments. In 2011 no hatching success experiment was run and the eggs collected were directly counted using a binocular microscope and fixed in 95% alcohol.

DNA amplification and sequencing

Adult individuals of the three *Acartia* species, *A. clausi*, *A. discaudata* and *P. grani* were prepared for PCR amplification by rehydrating in 22.75 µL DNA grade water in a PCR tube or 96-well plate at room temperature for between 4-6 h. Following this incubation 10 µL of 5x Flexi GoTAQ DNA polymerase buffer (Promega UK) was added to each sample and the sample homogenised using a hyperdermic needle (19G) inserted into a pellet pestle hand held homogeniser (Anachem Ltd) and incubated overnight at 4^{o}C. The remaining PCR reaction components were then added: 5 µL 2 mM dNTPS (Promega UK Ltd), 10 µM of primers LCO-1490 and HCO-2198 (LCO-1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO-2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', Folmer *et al.* 1994), 2 µL 25 mM $MgCl_2$ and 1.25 U GoTAQ DNA polymerase (Promega UK Ltd.). A positive control of *A. clausi* and *P. grani* extracted genomic DNA (100-200 ng) and a NTC (no template control) were included. Amplifications were carried out in a G Storm or VWR thermocycler. The cycling parameters included an initial denaturation step at 94.0^{o}C (4 min) followed by 40 cycles of 94.0^{o}C (1 min), 50.0^{o}C (1.5 min), 72.0^{o}C (1.5 min). A final extension phase at 72^{o}C (5 min) was followed by storage at 10.0^{o}C. 10 µL aliquots of the amplification reaction were analysed by gel electrophoresis (1%) to check amplification efficiency. 20 µL of successful amplifications were sent away for bidirectional sequencing by LGC Genomics GmbH, Germany. These sequences (n=71) were then used to perform a multiple sequence alignment using ClustalW analysis online at http://clustalW.genome.jp/ and

subsequently a consensus sequence for each species was constructed. The basic local alignment search tool (BLAST) was used to search the EMBL DNA database for sequence similarities. A FastA file of the sequences was converted to a sequence identity matrix using the BioEdit program (Hall 1999). To determine similarity and clustering of the sequences a nonmetric multidimensional scaling plot was constructed using PRIMER (Clarke 1993; Clarke and Warwick 2001).

Restriction mapping

The partial consensus sequences of mtCOI DNA for *A. clausi*, *A. discaudata* and *P. grani* were restriction mapped using the online NEBcutter V2.0 from New Englands BioLabs inc. (Vincze *et al*. 2003). From these restriction maps, suitable enzymes were selected to differentially digest each of the three species. To reduce both time and cost, the ability of the restriction enzyme to perform in the same buffer conditions as the amplification reaction were taken in to consideration.

Restriction digests

Restriction digests were performed on a 15 μL aliquot of PCR amplification product by the addition of 0.2 μL of BSA (10 μg mL^{-1}) and 5 U of each restriction enzyme (*Dde*I and *Pst*I Promega UK Ltd). Incubations were performed at 37^{o}C for 3-4 h. The digestion products were separated by gel electrophoresis on a 2% agarose gel and visualised on a UV transilluminator.

Molecular identification of *Acartia* eggs

Ethanol preserved

Following the development of the RFLP technique based on adults the method was then trialled on *Acartia* eggs collected from egg production experiments and then applied to the copepod eggs collected directly from the sediment. 24 eggs from core 1 (2-2.5 cm depth) collected in April 2011 were used to assess the ability of this technique to identify eggs from the sediment following the method described above.

During these studies, it became apparent that *Acartia* eggs, either from egg production experiments or from sediment, are poorly preserved in ethanol for any length of time. Therefore, the method was adapted to ensure successful amplification of DNA following preservation at -20^{o}C. This method is described below.

Preserved at -20^{o}C

Eggs were washed into a Petri dish with filtered seawater (fsw). Under a microscope a single egg was aspirated with 0.5 uL fsw and transferred to a new Petri dish containing 5 mL of ultrapure water (MilliQ) to "wash" the egg. The egg was then quickly (to prevent rupturing) aspirated with 23 uL of the ultrapure water and transferred into a well of a 96-well plate. After 4-6 hours, the plate was sealed and frozen at -20°C overnight or longer. The samples were then defrosted and the remaining PCR reaction components added to each well: 10 μL of 5x Flexi GoTAQ DNA polymerase buffer (Promega UK), 5 μL 2 mM dNTPS (Promega UK Ltd.), 10 μM of primers LCO-1490 and HCO-2198 (LCO-1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO-2198 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', Folmer *et al* 1994), 2 μL 25 mM $MgCl_2$ and 1.25 U GoTAQ DNA polymerase (Promega UK Ltd). Amplifications and restriction digests were then carried out as described above.

Results

A partial region (709 bp) of the mtCOI gene was successfully amplified directly from ethanol preserved individuals of the three *Acartia* spp. *A. clausi, A. discaudata* and *P. grani* (Figure 1) collected from Thau lagoon, France. In total 71 sequences were generated for this region of the mtCOI gene; *A. clausi* (n=22; 5 haplotypes; Accession numbers HE 863718-HE 863722), *A. discaudata* (n=21; 9 haplotypes; Accession numbers HE 863723-HE 863731) and *P. grani* (n=28; 10 haplotypes; Accession numbers HE 863732-HE 863741). A consensus sequence for each species was constructed (Figure 2). When compared to sequences on the EMBL DNA database, *A. clausi* sequences showed between 96%-100% identity over 85% coverage to existing sequences of *A. clausi* (Accession numbers: HE6647790.1-HE6479.1). No sequence homology greater than 85% was seen for sequences of either *P. grani* or *A. discaudata*. Over the amplified partial region of the mtCOI gene, the intraspecific variation ranged from ~2.0% for *A. discaudata*, to ~3.7% for *P. grani* and ~4.0% for *A. clausi*. The interspecific variation between these species was 29% (Figure 3).

The sequenced mtCOI regions for each of the three *Acartia* species were mapped and analysed to ascertain suitable restriction enzyme sites such that each species, when digested, would produce a unique restriction profile (Figure 4). The restriction enzymes *Dde*I and *Pst*I were chosen as they both produced a unique restriction profile for each species and because the enzymes exhibit optimum activity at the same temperature (37^{o}C) such that restriction digests can be performed in the same buffer conditions as the amplification reaction (Tritle 2006). Successful amplifications from individual *Acartia* adults and subsequent RFLP analysis resulted in a characteristic restriction profile or fingerprint for each species (Figure 5), thus allowing identification of *Acartia* species to species level.

Amplification of the mtCOI partial gene fragment was also successful from eggs collected from *Acartia* egg production experiments preserved in 95% ethanol and from eggs collected from sediment samples preserved in 95% ethanol. Of the 24 eggs processed from

sediment samples (Core 1, 2-2.5cm depth, April 2011) 15 amplified successfully. Of these successful amplicons all 15 digested to produce a restriction profile for *A. clausi* (n=5) or *P. grani* (n=10). In order to confirm the accuracy of species identification by RFLP on sediment eggs the amplified products were sequenced. In all cases the sequences confirmed the species identity of the individual eggs to be the same as those determined by RFLP. During this study it became apparent that the eggs were not stable stored in ethanol for any length of time. After six months it was no longer possible to successfully amplify the DNA from individual eggs. As such the technique was adapted to provide a protocol for future preservation of individual eggs that is robust and reliable. The technique described was trialled on *A. clausi* eggs laid during egg production experiments and routinely allowed amplification of individual eggs with a success rate of over 80%.

Discussion

A simple molecular method involving the direct amplification of a partial region of the gene encoding the mitochondrial protein cytochrome oxidase subunit I (mtCOI) and subsequent restriction fragment length polymorphism (RFLP) has been developed to identify individuals of *A. clausi*, *A. discaudata* and *P. grani*. In order to develop this technique a total of 71 sequences were generated for this region of the mtCOI gene for all three congeneric species collected from the Thau lagoon, France. Over the amplified partial region of the mtCOI gene the intraspecific variation ranged from ~2.0% for *A. discaudata*, to ~3.7% for *P. grani* and ~4.0% for *A. clausi*, an accepted level of intra-specific difference for this gene region. Hill *et al*. (2001) found intraspecific variation of between 0.5% and 2.8% for this region of gene in 10 *Calanus* species. For the same region of gene intra-specific variation was less than 2% for *Pseudocalanus* species (Grabbert *et al*. 2010) and between 0.5% and 4.2% for 13 species of *Clausocalanus* (Bucklin *et al*. 2010). When compared against sequences on

the EMBL DNA database *A. clausi* showed between 96% and 100% homology with existing *A. clausi* sequences, further helping to confirm the identity of our species. No homology for sequences of either *P. grani* or *A. discaudata* over 85% similarity was seen. In the case of *A. discaudata* this is understandable as there are no existing COI sequences for this species on the database. There is however, a recent submission of a putataive *P. grani* COI sequence from the Egyptian coast of the Mediterranean Sea (Accession number JQ245071.1) on the database. When aligned with our *P. grani* sequences it showed a variation greater than 30%. This intra specific variation is extremely high for this region of gene, as discussed above the range of intra specific variation is usually between 0% and 4%. It is impossible to explain this high level of variation between the sequences of *P. grani* from the Thau lagoon, France and the *P. grani* sequence from the Egyptian coast of the Mediterranean Sea as the latter is only a single unverified sequence.

The inter-specific variation between these congeneric *Acartia* species was 29%. This is also an accepted level of variation for this gene region between congeneric species. Previous studies have shown pair wise sequence differences to be between ~18% for two species of *Pseudocalanus* (Bucklin *et al*. 1998), between 7.4% and 25.7% for 10 congeneric species of *Calanus* (Hill *et al*. 2001) and between 8.6% and 24.9% for 13 species of Clausocalanus (Bucklin *et al*. 2010).

The developed technique is capable of determining the identity of individual copepods and individual eggs, including resting eggs retrieved from sediment samples. Consideration has been made to produce a reliable, quick and cost effective technique. As such amplification is carried out directly on preserved samples negating the need for prior purification of the DNA. While PCR products can be purified prior to restriction endonuclease digestion, both time and reagents are saved by performing restriction digests in the same buffer conditions as the amplification reaction. Eliminating a purification step after amplification and before

252 restriction digest also eliminates potential pipetting error and decreases assay preparation
253 time.

254 Amplification of adults preserved in 95% ethanol was consistently successful and
255 unproblematic. The success rate of amplification from eggs however, was much lower than
256 for adults. Initially this was thought to be because of the very small size of the eggs
257 (~74.4±1.5 µm for *A. clausi*; Belmonte 1998) which poses considerable challenges when
258 manipulating them into a PCR tube or 96-well plate. This problem is exasperated when the
259 eggs have been stored in ethanol as they then appear to be 'static' and regularly stick to all
260 plastic-ware. Further tests revealed that the eggs ruptured on contact with the ethanol and that
261 the cytoplasm burst through the membrane of the egg. It was possible to successfully amplify
262 DNA from the eggs that had been stored in ethanol if the preservation was under 6 months.
263 However, the eggs appear to be unstable in the ethanol and DNA amplification was
264 impossible after a relatively short period of time (~ six months). As such a new protocol has
265 been developed whereby eggs, originating from the water column, egg production
266 experiments or sediment, are washed in filtered sea water and then rinsed in ultra-pure MilliQ
267 water. The eggs are then removed with the correct volume of water for the PCR reaction and
268 isolated in a well of a 96-well plate before they rupture. This protocol has many benefits. As
269 the eggs have not been stored in ethanol they are not static, which makes handling much
270 easier. The eggs are isolated in individual wells before rupturing thus preventing any possible
271 cross contamination, the technique significantly reduces the time consuming and difficult
272 manipulation of the eggs compared to storage of multiple eggs in a single vial, and perhaps
273 most importantly, it allows successful amplification of individual eggs.

274 For the first time a technique now exists to identify the 3 congeneric species of co-
275 occurring Acartia; *A. clausi*, *A. discaudata* and *P. grani* from the Thau lagoon, France. This
276 technique can identify individuals from eggs to adults including resting eggs found in the

sediment. To our knowledge this is the first time that resting eggs in the Thau lagoon have been identified to species level. The developed technique was trialled on a small sample of resting eggs retrieved from the sediment and showed the presence of both *A. clausi* and *P. grani* species. These results confer with the literature that both these species are able to produce resting eggs (i.e. Kasahara *et al* 1974; Marcus 1990; Guerrero and Rodríguez 1998). Care must be taken however, as the COI primers used are universal and may therefore amplify COI from other organisms. In the Thau lagoon the resting eggs are easily identifiable as copepod eggs, and although there is a slight chance that other copepods found in the lagoon may produce resting eggs this is firstly unlikely (undocumented for all but *Eurytemora velox*) and secondly these species such as *Eurytemora* and *Centropages* species make up only a very small proportion of the total zooplankton community in the lagoon (<10%) (Lam Haoi 1985).

Considering the difficulties of identifying early developmental stages to species using morphological characters and the time constraints of hatching resting eggs and culturing them to maturity in order to allow identification, this new method will allow better population dynamic studies to be performed on these important and dominant members of the zooplankton community in the Thau lagoon, France.

Acknowledgements:

The present study was supported by the GELAMED project through a grant allocated by the MEEDDM and the Total foundation to D. Bonnet (Programme 189 – « Recherche » 18902 C).

References:

Alcaraz M (1983) Coexistence and segregation of congeneric pelagic copepods: spatial distribution of the *Acartia* complex in the Ría of Vigo (NW of Spain). J Plankton Res 6: 891-900

303 Arfi R, Champalbert G, Patriti G (1981) Système planctonique et pollution urbaine: un aspect
304 des populations zooplanctoniques. Mar Biol 61: 133-141
305 Belmonte G (1998) The egg morphology of 7 Acartiidae species: a preliminary survey of the
306 ootaxonomy of calanoids. J Mar Syst 15: 35-39
307 Blanco-Bercial L, Álvarez-Marqués F (2007) RFLP procedure to discrimínate between
308 *Clausocalanus* Giesbrecht, 1888 (Copepoda, Calanoida) species in the Central
309 Cantabrian Sea. J of Exper Mar Biol and Ecol 344: 73-77
310 Boyer S, Arzul I, Bonnet D (In press) Some like it hot: *Paracartia grani* (Copepoda:
311 Calanoida) arrival in the Thau lagoon (South of France- Mediterranean Sea). Marine
312 Biodiversity Records
313 Boyer S, Bouvy M, Bonnet D (submitted) What does trigger *Acartia* species egg production
314 and hatching success in a Mediterranean lagoon? Estuar Coast Shelf Sci
315 Bucklin A, Bentley M, Franzen S (1998) Distribution and relative abundance of
316 *Pseudocalanus moultoni* and *P. newmani* (Copepoda:Calanoida) on Georges Bank using
317 molecular identification of sibling species. Mar Biol 132: 97-106
318 Bucklin A, Guarnieri M, Hill R, Bentley A, Kaartvedt S (1999) Taxonomic and systematic
319 assessment of planktonic copepods using mitochondrial COI sequence variation and
320 competitive, species-specific PCR. Hydrobiologia 401: 239-254
321 Bucklin A, Hopcroft R, Kosobokova K, Nigro L, Ortman B, Jennings R, Sweetman C (2010)
322 DNA barcoding of Arctic Ocean holozooplankton for species identification and
323 recognition. Deep-Sea Res Part II- Topical Studies in Oceanography 57(1-2): 40-48
324 Calbet A, Saiz E, Irigoien X, Alcaraz M, Trepat I (1999) Food availability and diel feeding
325 rhythms in the marine copepods *Acartia grani* and *Centropages typicus*. J Plankton Res
326 21(5): 1009-1015
327 Clark KR (1993) Non-parametric multivariate analyses of changes in community structure.
328 Aust Journal of Ecol 18: 117-143

329 Clarke KR, Warwick RM (2001) Change in marine communities: an approach to statistical
330 analysis and interpretation, 2nd edition. PRIMER-E, Plymouth
331 Fatemi M (1938) Les variations saisonnières du plancton de l'Etang de Thau à l'embouchure
332 du canal de Sète. PhD Thesis Université Montpellier 2 (France) 97 pp.
333 Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (2004) DNA primers for amplification of
334 mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates.
335 Molec Mar Biol and Biotechnology 3(5): 294-299
336 Grabbert S, Renz J, Hirche H-J, Bucklin A (2010) Species-specific PCR discrimination of
337 species of the calanoid copepod *Pseudocalanus*, *P. acuspes* and *P. elongatus*, in the
338 Baltic and North Seas. Hydrobiologia 652(1): 289-297
339 Guerrero F (1993) Producción, estrategias reproductivas y proliferación de *Acartia*
340 (Copepoda: calanoida) en sistemas costeros sometidos a distintas escalas temporales
341 de fluctuación ambiental. PhD Tesis Universidad de Málaga 206 pp.
342 Guerrero F, Rodríguez V (1998) Existence and significance of *Acartia grani* resting eggs
343 (Copepoda: Calanoida) in sediments of a coastal station in the Alboran Sea (SE Spain). J
344 Plankton Res 20(2): 305-314
345 Hall T A (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis
346 program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95-98
347 Hill RS, Allen LD, Bucklin A (2001) Multiplexed species-specific PCR protocol to
348 discriminate four N.Atlantic *Calanus* species, with an mtCOI gene tree for ten *Calanus*
349 species. Mar Biol 139: 279-287
350 Kasahara S, Uye S and Onbé T (1974) Calanoid copepod eggs in Sea-Bottom Muds. Mar Biol
351 26: 167-171

352 Lakkis S (1994) Coexistence and competition within *Acartia* (Copepoda, Calanoida)
353 congeners from Lebanese coastal water: niche overlap measurements. Hydrobiologia
354 292/293: 481-490
355 Lam Haoi T (1985) Evolution saisonnière du zooplancton dans trois sites peu profonds de
356 Thau, une lagune Nord-Méditerranéenne. Hydrobiologia 128: 161-174
357 Lindeque PK, Harris RP, Jones MB, Smerdon GR (1999) Simple molecular method to
358 distinguish the identity of *Calanus* species (Copepoda: Calanoida) at any developmental
359 stage. Mar Biol 133 :91-96
360 Mathias P, Euzet L (1962) Le plancton du bassin de Thau (Etang des Eaux Blanches).
361 Naturalia Monspeliensia IV: 7-28
362 Marcus NH (1990) Calanoid copepod, cladocerans, and rotifer eggs in sea-bottom sediments
363 of northern California coastal waters: identification, occurrence and hatching. Mar Biol
364 105: 413-418
365 Onbé T (1978) Sugar flotation method for sorting the resting eggs of marine cladocerans and
366 copepods from sea-bottom sediment. Bulletin of Japanese Society of Scientific Fisheries
367 44 (12): 1411
368 Rose M (1933) Copépodes pélagiques. In: Faune de France (26): 374 pp
369 Tritle D (2006) Activity of Promega Restriction Enzymes in GoTaq® Green and PCR Master
370 Mixes. Promega Corporation Web site.
371 http://www.promega.com/resources/articles/pubhub/enotes/activity-of-promega-
372 restriction-enzymes-in-gotaq-green-and-pcr-master-mixes/ Updated 2006. Accessed 11th
373 Jan 2012
374 Vincze T, Posfai J, Roberts RJ (2003) NEBcutter: a program to cleave DNA with restriction
375 enzymes. Nucleic Acids Res 31: 3688-3691

376 Webb K, Barnes D, Clark M, Bowden D (2006) DNA barcoding: A molecular tool to identify
377 Antarctic marine larvae. Deep-Sea Res Part II-Topical studies in Oceanogr 53(8-10):
378 1053-1060

379 Yamazi I (1964) Structure of the netted plankton communities in the inner area of the Gulf of
380 Naples in September 1962. Publ Staz Zool Napoli 34: 98-136

381

382

383

384

Figure Legends:

Fig. 1 Amplification of a 709 base pair region of the mtCOI gene from preserved adult *Acartia* M = 100bp DNA ladder, lanes 1-12 = *A. clausi*, lanes 13-24 = *P. grani*, lanes 25-36 = *A. discaudata*. –ve = no DNA template control, +ve = positive control of *A. clausi* and *P. grani* extracted genomic DNA (100-200ng)

Fig. 2 Consensus sequence of a 709 bp region of the mtCOI gene for the three species *A. clausi* (*clausi*), *A. discaudata* (*discaudata*) and *P. grani* (*grani*). * = homology between all three species; **ctnag** = *Dde*I restriction site; **ctgcac** = *Pst*I restriction site; primers in italics

Fig. 3 Nonmetric multidimensional scaling plot demonstrating similarity and clustering of *A. clausi* ▲, *P. grani* ▼ and *A. discaudata* ■. All sequences (n=71) were used to create a sequence identity matrix. Sequences from the same species cluster with >96% similarity ------ A. *clausi* and *A. discaudata* cluster at >80% similarity ------ and all three species cluster at >70% similarity ——

.

Fig. 4 Restriction map of a partial region of the mtCOI gene for *A. clausi*, *P. grani* and *A. discaudata*. *Dde*I and *Pst*I restriction sites are indicated. Resulting fragment lengths are shown in base pairs (bp)

Fig. 5 Amplification of a region of mtCOI gene from whole *Acartia* adults and subsequent RFLP analysis with restriction enzymes *Dde*I and *Pst*I. M=100bp DNA markers, Lanes 1-3 = restriction profile for *P. grani*; Lanes 4-6 restriction profile for *A. discaudata*; Lanes 7-9 = restriction profile for *A. clausi*; Lane 10 = undigested amplicon

Figure

Fig. 1

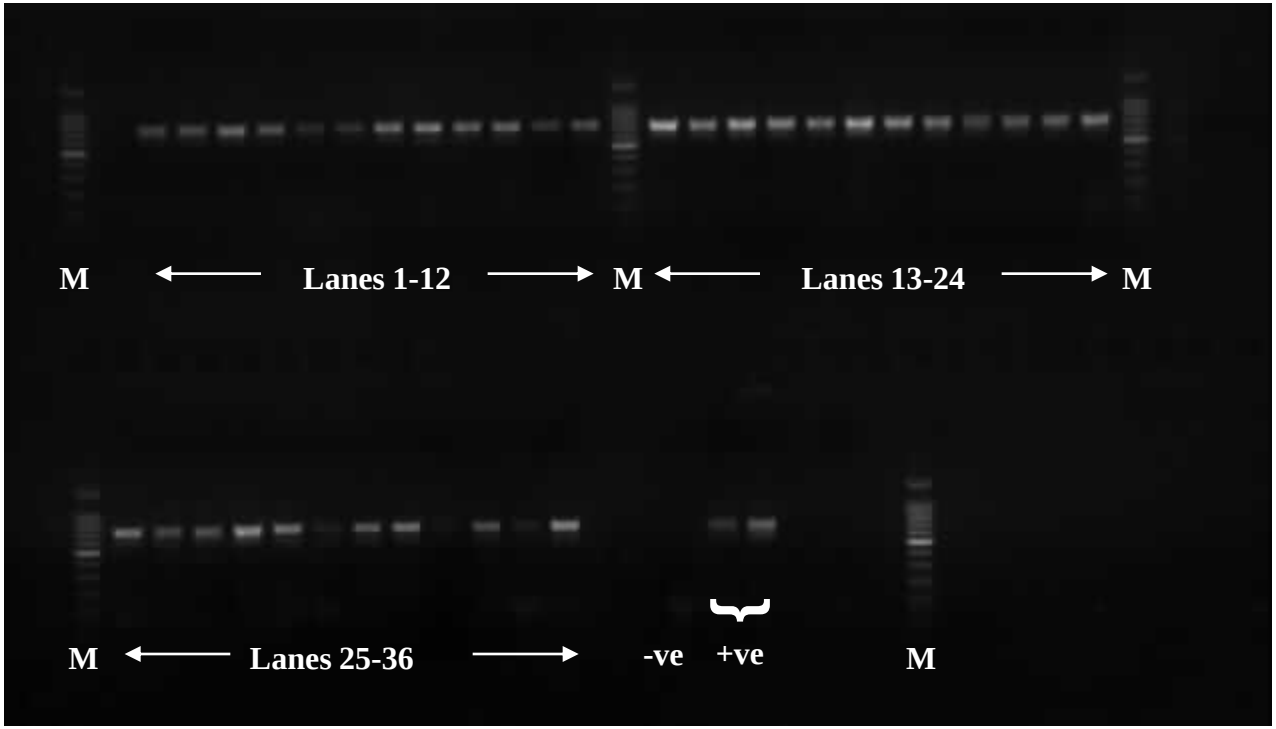


Fig. 1 Amplification of a 709 base pair region of the mtCOI gene from preserved adult *Acartia* M = 100bp DNA ladder, lanes 1-12 = *A. clausi*, lanes 13-24 = *P. grani*, lanes 25-36 = *A. discaudata*. –ve = no DNA template control, +ve = positive control of *A. clausi* and *P. grani* extracted genomic DNA (100-200ng)

Figure

Fig. 2

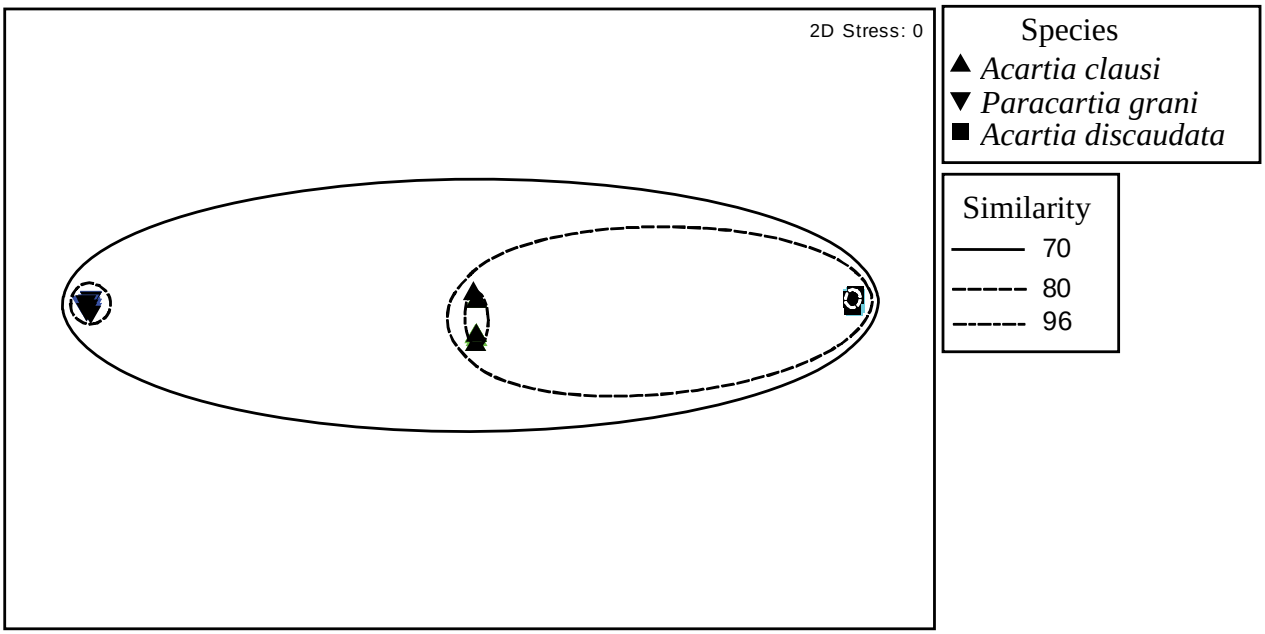
```
clausi          GGTCAACAAATCATAAAGATATTGGCACTTTATATTTACTRGCTGGTATRTGGTCGGGGA
discaudata      GGTCAACAAATCATAAAGATATTGGYACTCTYTACCTTTTAGCTGGGATGTGGTCAGGAA
grani           GGTCAACAAATCATAWAGATATTGGGACTTTATATTTATTAGCAGGGSCTTGATCTGGAA
                *************** ********* *** * **  *  * ** **    ** ** ** *

clausi          TAGTRGGCACTGGATTAAGAATRATCATYCGAATRGAGCTTGGTCAAGCAGGAAAATTAA
discaudata      TGGTGGGAACTGGCTTGAGAATGATCATTCGGATAGAAYTAGGCCAAGCTGGAAAATTAA
grani           TAGTTGGAACAGGCCTTAGAATAATTATTCGATTAGARCTGGGTCAAGCTGGAAGATTAA
                * ** ** ** **  * ***** ** ** **  * **  * ** ***** **** *****

clausi          TTGGAGATGAYCAAATYTATAACGTAGTTGTAACAGCCCATGCCTTTATTATAATTTTTT
discaudata      TTGGGGATGATCAAATTTATAACGTGGTCGTTACAGCCCACGCGTTTATTATAATTTTCT
grani           TTGGAGATGATCAAATTTACAATGTTGTTGTAACCGCTCATGCYTTTATTATAATTTTTT
                **** ***** ***** ** ** ** ** ** ** ** ** ** ************** *

clausi          TTATAGTTATACCAATTCTAATTGGRGGGTTTGGRAATTGAYTAATYCCCCTAATACTAG
discaudata      TTATAGTAATACCTATTTTAATTGGGGGGTTTGGAAATTGACTAATCCCTCTTATACTWG
grani           TCATAGTYATACCTATTCTAATTGGAGGATTTGGRAATTGACTAGTGCCTCTTATATTGG
                * ***** ***** *** ******* ** ***** ****** ** * ** ** *** * *

clausi          GAGCTGCAGAYATAGCYTTCCCYCGAATAAATAATATAAGGTTTTGRCTCCTACTTCCTG
discaudata      GGGCTGCTGACATAGCRTTTCCCCGCATAAATAATATAAGATTTTGACTCCTACTCCCCG
grani           GTGCAGCGGATATAGCATTCCCTCGAATAAATAATATAAGATTTTGATTCTTAATACCAG
                * ** ** ** ***** ** ** ** ************** *****  ** ** * ** *

clausi          CTTTAGTYATGCTTTTATCTAGTTCTTTAGTGGAAAGAGGGGCAGGAACTGGGTGAACAG
discaudata      CCTTGATTATRTTGCTGTCAAGRTCCTTAGTGGAAAGGGGGGCTGGRACCGGCTGAACTG
grani           CTTTAATTATATTRYTATGTAGTTCATTAGTTGAAAGGGGRGCCGGAACAGGTTGAACAG
                * **  * **  *  * *  ** ** ***** ***** ** ** ** ** ** ***** *

clausi          TTTAYCCACCMCTATCTAGAAATATTGCCCATGCAGGRGCTTCAGTTGATTTCGCAATTT
discaudata      TTTACCCCCCCCTCTCAAGCAATATTGCCCACGCAGGCAGGGCTGTAGATTTTGCAATTT
grani           TGTAYCCCCCTTTGTCGAGGAAYATTGCTCATGCGGGAGCTTCAGTGGATTTTGCTATTT
                * ** ** **  * ** ** ** ***** ** ** **     * ** ***** ** ****

clausi          TTTCCCTCCATCTTGCAGGTGCAAGATCTATTTTAGGAGCTGTAAACTTTATYTCTACTG
discaudata      TTTCACTCCATTTAGCGGGRGCCAGTTCTATTCTAGGAGCTGTAAATTTTATTTCTACTA
grani           TTTCTTTACATCTTGCGGGTGTAAGTTCTATTTTAGGKGCTGTTAATTTTATCTCAACTG
                ****  * *** * ** ** *  ** ****** **** ***** ** ***** ** ***

clausi          TAGGWAACCTTCGATCATTTGGGATAATAGCGGATTTAATACCTTTRTTTAGGTGGGCGG
discaudata      TTGGTAACTTGCGATCATTTGGCATGATAGCGGATCTAATACCTTTGTTTAGRTGGGCTG
grani           TTGGAAATCTTCGGGCATTTGGAATAATTCTTGATCGAATACCATTATTTGCATGAGCAG
                * ** **  * **  ******* ** **    ***  ****** ** ***   ** ** *

clausi          TATTAATTACAGCTGTTTTACTACTYTTATCTTTGCCAGTRTTAGCGGGGGCTATYACTA
discaudata      TGGTAATTACAGCGGTTTTGCTTTTATTATCTCTTCCTGTTTTAGCCGGGGCTATTACTA
grani           TATTAATTACAGCAGTTCTATTRCTATTRTCTTTACCTGTAYTAGCTGGGGCTATTACTA
                *  ********** *** *  *  * ** *** * ** **  **** ******** ****

clausi          TACTTCTCACTGATCGTAATTTAAACTCTTCTTTTTATGACGCCTGCGGTGGAGGAGAYC
discaudata      TGCTTTTAACYGACCGTAATTTGAATTCATCGTTTTACGATGCYGGAGGGGGGGGAGAYC
grani           TATTATTRACTGAYCGTAACTTAAATTCATCATTCTACGATGCTAGAGGKGGCGGCGACC
                *  *  * ** ** ***** ** ** ** ** ** ** ** **  * ** ** ** ** *

clausi          CYATCCTTTATCAACATTTATTTTGATTTTTTGGTCACCCTGAAGTTTA
discaudata      CYATCCTGTATCAGCACCTGTTTTGATTTTTTGGTCACCCTGAAGTTTA
grani           CTATCTTRTATCAGCATTTATTTTGATTTTTTGGTCACCCTGAAGTTTA
                * *** * ***** **  * ****************************
```

Fig. 2 Consensus sequence of a 709 bp region of the mtCOI gene for the three species *A. clausi* (*clausi*), *A. discaudata* (*discaudata*) and *P. grani* (*grani*). * = homology between all three species; **ctnag** = *Dde*I restriction site; **ctgcac** = *Pst*I restriction site; primers in italics

Figure

Fig. 3 Nonmetric multidimensional scaling plot demonstrating similarity and clustering of *A. clausi* ▲ *P. grani* ▼and *A. discaudata* ■. All sequences (n=71) were used to create a sequence identity matrix. Sequences from the same species cluster with >96% similarity ------ *A. clausi* and *A. discaudata* cluster at >80% similarity ------and all three species cluster at >70% similarity ——

Fig. 3



Figure

Fig 4

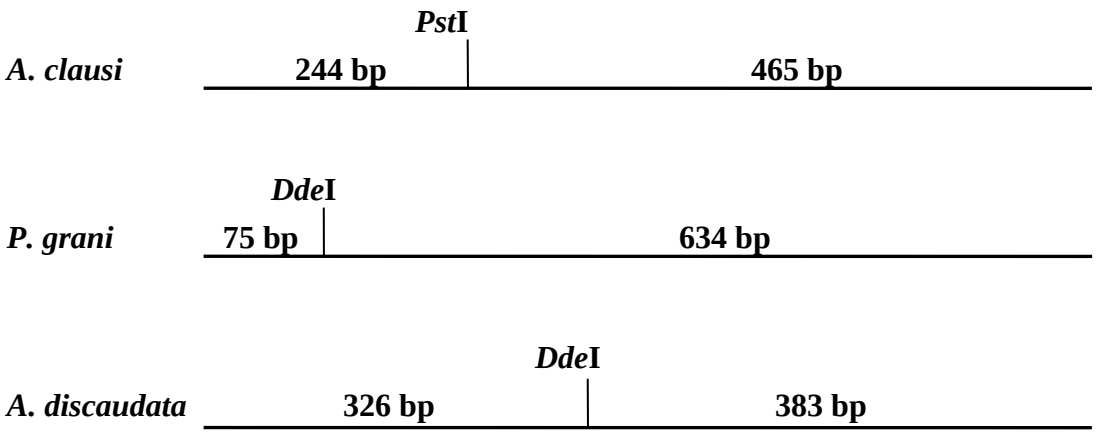


Fig. 4 Restriction map of a partial region of the mtCOI gene for *A. clausi*, *P. grani* and *A. discaudata*. *Dde*I and *Pst*I restriction sites are indicated. Resulting fragment lengths are shown in base pairs (bp)

Figure

Fig. 5

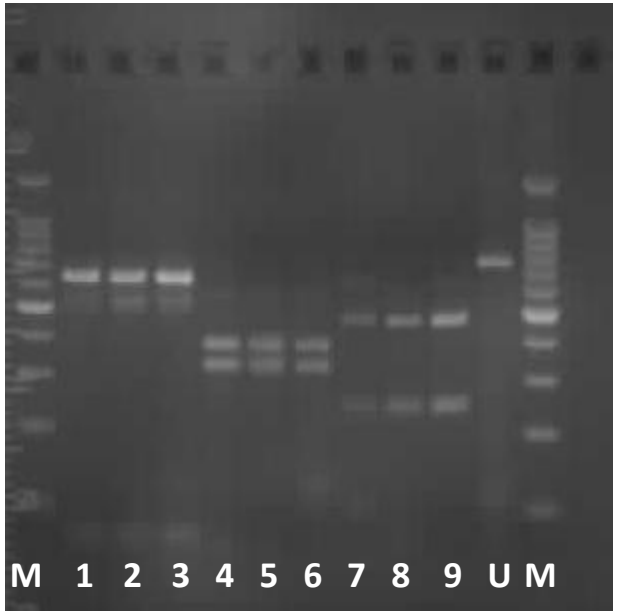


Fig. 5 Amplification of a region of mtCOI gene from whole *Acartia* adults and subsequent RFLP analysis with restriction enzymes *Dde*I and *Pst*I. M=100bp DNA markers, Lanes 1-3 = restriction profile for *P. grani*; Lanes 4-6 restriction profile for *A. discaudata*; Lanes 7-9 = restriction profile for *A. clausi*; Lane 10 = undigested amplicon.